Control of Respiration and Nitrogen Fixation by Oxygen and Adenine Nucleotides in N₂-grown Azotobacter chroococcum

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SUMMARY

Oxygen uptake by Azobacter chroococcum (NCIB 8003) grown in continuous culture without fixed nitrogen and with a low mannitol concentration (2.5 g./l.) and treated with lysozyme and EDTA was inhibited by ATP but not by ADP; ADP frequently prevented inhibition by ATP. In preparations obtained by disrupting bacteria suspended in a mixture of defatted bovine serum albumin, sucrose and MgCl₂ in the French press, ATP inhibited oxygen uptake with either sodium succinate or sodium isocitrate as substrates and ADP prevented this inhibition; oxygen uptake with glucose-6-phosphate was inhibited by ATP or ADP. A form of respiratory control by nucleotides may thus occur in Azotobacter. Acetylene reduction (a measure of nitrogenase activity) by bacteria treated with lysozyme and EDTA was inhibited by ATP; this was attributed to inhibition of oxygen uptake by ATP causing inhibition of nitrogenase by oxygen. High oxygen solution rates inhibited nitrogenase in whole bacteria or in bacteria treated with lysozyme and EDTA; when the oxygen solution rate was lowered nitrogenase functioned immediately. These observations are probably expressions of processes which protect nitrogenase in whole bacteria from damage by oxygen.

INTRODUCTION

Inhibition by ATP of oxygen uptake and the prevention of this inhibition by ADP has been observed in mitochondria and described by Klingenberg & Schollmeyer (1960) and by Chance & Hollunger (1961). A similar type of respiratory control has not been found in bacteria, although stimulation of oxygen uptake by inorganic phosphate in particulate preparations from the following bacteria has been reported: Micrococcus lysodeikticus (Ishikawa & Lehninger, 1962); Alkaligenes faecalis (Scocca & Pinchot, 1965) and Mycobacterium phlei (Revsin & Brodie, 1967). Revsin and Brodie also reported that oxygen uptake was inhibited by ATP or ADP and stimulated by AMP in their preparations from M. phlei provided inorganic phosphate was present. Oxygen uptake by a particulate NADH oxidase from M. tuberculosis was stimulated by AMP or ADP; this stimulation did not depend upon added inorganic phosphate and was attributed to allosteric activation of the NADH oxidase by AMP (Worce, Goldman & Cleland, 1965).

Respiratory control by ATP in mitochondria depends upon a high degree of physical intactness (see Lehninger, 1964); it is possible that a similar degree of intactness is necessary to show respiratory control by ATP in bacteria. Inhibition by ATP of
oxygen uptake in N₂-grown Azotobacter chroococcum treated with lysozyme and ethylene diamine tetracetic acid (EDTA) is reported in the first part of this paper.

Experiments with cell-free extracts indicate that nitrogen fixation, even in anaerobic organisms, is a reductive process (see Postgate, 1969). Dalton & Postgate (1967) found that moderate oxygen tensions inhibited the growth of N₂-grown but not NH₄-grown Azotobacter chroococcum and they suggested that oxygen inhibited nitrogen fixation in the growing organism. Aerobic nitrogen fixing bacteria must, when growing, possess mechanisms that protect the nitrogenase from oxygen damage; Dalton & Postgate (1969) suggested two mechanisms: oxygen removal by respiration (Azotobacter has exceptionally high QO₂ values: Williams & Wilson, 1954) and a conformational state that protects the nitrogenase from oxygen damage. Nitrogenase reduces acetylene to ethylene (Dilworth, 1966, Schollhorn & Burris, 1967); this reaction can be used as a sensitive assay of nitrogenase activity. The action of ATP on acetylene reduction, together with its effect on oxygen uptake, by A. chroococcum grown in continuous culture, is described in the second part of this paper.

 METHODS AND MATERIALS

Growth of organism. Azotobacter chroococcum (NCIB 8003) was grown in continuous culture (Baker, 1968) using a nitrogen-free medium containing 2·5 g. mannitol/l. and the trace elements prescribed by Dalton & Postgate (1969) at a dilution rate of 0·1 hr⁻¹ at 30°C. The culture had a working volume of approximately 500 ml. and was aerated by a 4 cm. magnetic stirring bar at 300 rev./min. under a stream of air (800 ml./min.). Under these conditions the population in the culture was oxygen-limited.

Acetylene reduction by whole bacteria or by bacteria treated with lysozyme and EDTA. Nitrogenase activity in growing Azotobacter chroococcum was very sensitive to environmental change: for example, organisms allowed to stand unaerated at room temperature for 5 min. showed severalfold less activity than organisms that were tested immediately after sampling; J. Drozd & J. R. Postgate (personal communication) observed a comparable ‘switch-off’ of activity which they attribute to aeration. Care was therefore taken that culture samples were handled identically throughout. Even so, no significance was attached to different acetylene reduction rates between experiments and only complete inhibition of acetylene reduction was accorded significance within experiments.

Acetylene reduction and oxygen uptake were assayed with 10 ml. samples from the continuous culture injected immediately after sampling, without aseptic precautions, through a side-arm into 50 ml. double side-armed Warburg manometer flasks stoppered with ‘Suba Seal’ closures, and containing the atmospheres and reagents described under Table 2 and Fig. 1, 3 and 4. The flasks and reagents were pre-incubated at 30° for 10 min. before adding the culture. Acetylene (1·0 ml.), produced by adding water to CaC₂, was added last; setting up took 5 to 10 sec. from the start of sampling the culture. Oxygen uptake was measured manometrically: 2 ml. gas samples from the manometer flasks (replaced with air pre-incubated at 20°) were assayed for ethylene by vapour phase chromatography on a 5 ft column of Porapak R (4 mm. internal diameter) at 45° with N₂ (50 ml./min.) as the carrier gas using a Pye 104 gas chromatography instrument with a flame ionization detector head.

Mechanically disrupted preparations. Azotobacter chroococcum (300 ml.) was
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harvested at 30,000 g for 15 min. and resuspended in 3 ml. of 50 mM-tris (hydroxymethylaminomethane buffer (pH 7.4), containing 2% (w/v) bovine serum albumin, 0·3 M-sucrose and 0·1 M-MgCl₂ (a mixture that protects oxidative phosphorylating systems in adipose tissue: Aldridge & Street, 1968) and disrupted in the French pressure cell from 8000 lb./sq. in. Preparations contained approximately 5 mg. of bacterial protein/ml. Oxygen uptake in these preparations was measured manometrically or with a Clark oxygen electrode.

Dehydrogenase activities. These were measured by the rate of reduction of tetrazolium salt (Fahmy & Walsh, 1952): assays contained tetrazolium (2·4 mg.) in 50 mM-tris buffer (pH 7·4) with substrate (50 μmoles) and protein (0·5 mg.) and were run under argon at 30° in a shaker.

P/O ratios. These were determined by measuring the oxygen uptake and the esterification of inorganic phosphate in the presence of an ATP trap (glucose and hexokinase) over 20 min. Esterification of inorganic phosphate was measured as the amount of radioactive 32P incorporated according to the method of Nielsen & Lehniger (1955). The radioactivity was measured with an IDL end window counter (lead castle type 710) at 540 volts.

Cytochrome spectra. These were measured in an oxygen atmosphere at room temperature on a Unicam SP 700 using a reflectance head. Approximately 2·4 mg. bacterial protein/ml. 0·1 M-tris (pH 7·4) were used.

Materials. All biochemicals, including hexokinase (E.C.2.7.1.1) and lysozyme (E.C.3.2.1.17) were purchased from Sigma Chemical Co. (London) Ltd.

RESULTS

Effects of nucleotides on oxygen uptake

Whole bacteria. ATP or ADP had no effect on oxygen uptake in whole organisms (Fig. 1 b).

Bacteria treated with lysozyme and EDTA. ATP inhibited oxygen uptake but ADP did not (Fig. 1 a). In some experiments ADP stimulated oxygen uptake while in 60% of cases equimolar ADP wholly, or partly, prevented inhibition by ATP.

Disrupted preparations. NADH and sodium succinate were used as key substrates to the respiratory chain to test the effect of adenine nucleotides on oxygen uptake with disrupted preparations. Two other dehydrogenases reported to be affected by adenine nucleotides were also tested: glucose-6-phosphate dehydrogenase (this enzyme, from several organisms, was inhibited by ATP; Schindler & Schlegel, 1969); iso-citric dehydrogenase (in mammalian systems this enzyme is inhibited by ATP and stimulated by ADP; see Mahler & Cordes, 1966). NADH and glucose-6-phosphate dehydrogenases were the two most active in disrupted preparations, reducing tetrazolium chloride five to 10 times as rapidly as succinic, isocitric and other dehydrogenases. The effects of ATP, ADP and phosphate ions on oxygen uptake with these four substrates are shown in Table 1. NADH oxidase appeared to be unaffected by ATP, ADP or a mixture of both over 20 min. However, observations with manometric experiments suggested that oxygen uptake in the first 3 min. was inhibited by ATP and this inhibition was prevented by ADP. When the system was examined with the oxygen electrode ATP completely inhibited oxygen uptake only over the first 30 sec. (Fig. 2). The presence of 5 mM-KF extended the inhibition by ATP to between 1 and
2 min. Oxygen uptake with succinate or with isocitrate was partly inhibited, usually between 20 and 50 %, by ATP; this effect was prevented by ADP. Glucose-6-phosphate dehydrogenase was inhibited by ATP, ADP or inorganic phosphate. Inorganic phosphate had no effect upon oxygen uptake with any of the other three substrates.

Table 1. Effect of adenine nucleotides on oxygen uptake with disrupted preparations from Azotobacter chroococcum

<table>
<thead>
<tr>
<th>Substrate (sodium salts)</th>
<th>Glucose-6-phosphate</th>
<th>Isocitrate</th>
<th>Succinate</th>
<th>NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Additions)</td>
<td>6.6</td>
<td>5.8</td>
<td>6.1</td>
<td>6.9</td>
</tr>
<tr>
<td>ATP</td>
<td>3.25</td>
<td>4.4</td>
<td>4.8</td>
<td>6.5</td>
</tr>
<tr>
<td>ADP</td>
<td>3.4</td>
<td>5.7</td>
<td>6.8</td>
<td>7.25</td>
</tr>
<tr>
<td>AMP</td>
<td>3.6</td>
<td>6.0</td>
<td>6.4</td>
<td>6.8</td>
</tr>
<tr>
<td>ATP + ADP</td>
<td>2.3</td>
<td>5.2</td>
<td>5.6</td>
<td>7.25</td>
</tr>
<tr>
<td>Phosphate ions</td>
<td>2.15</td>
<td>5.7</td>
<td>5.7</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Conditions: Experiments were run in 15 ml. Warburg manometer flasks at 30°C under air in water (2.4 ml) containing Tris buffer (pH 7.4) (120 μmoles) NAD (1 μmole) and disrupted A. chroococcum in the following concentrations: 0.75 mg. and 0.83 mg. with isocitrate or succinate as substrate, 0.3 mg. and 0.28 mg. with glucose-6-phosphate or NADH. Substrates (50 μmoles, except NADH, 10 μmoles) and nucleotides (10 μmoles) or phosphate ions (50 μmoles) were added from the side-arm to start the experiment. The centre well contained 40% KOH (0.1 ml.).
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\textit{Esterification of inorganic phosphate.} \textit{Azotobacter chroococcum} treated with lysozyme and EDTA esterified inorganic phosphate; this esterification was stimulated approximately 2-fold by added ADP (10 \(\mu\text{moles}\)) although no significant increase in oxygen uptake occurred. The highest P:O ratio was 0.25 measured from the total oxygen uptake and total esterification with ADP. Disrupted preparations also esterified inorganic phosphate with a P:O ratio of 0.52. It seems unlikely at face value that mechanically disrupted cells should yield higher P:O ratios than cells suffering more gentle damage by lysozyme and EDTA. Perhaps some of the oxygen was taken up by cells that were not sufficiently damaged to allow access of ADP; this would yield an exaggerated oxygen uptake compared with the stimulation of phosphate esterification by ADP.

\textit{Effects of nucleotides on cytochrome spectra.} The effect of ATP and/or ADP on the steady level of cytochromes in disrupted preparations was measured with NADH or sodium succinate as the electron donor. ATP (20 \(\mu\text{moles}\)) caused up to 20\% reductions in the \(\alpha\) and \(\beta\) peaks of the \(b\) and \(c\) cytochromes; ADP did not have this effect. Adding ADP to preparations to which ATP had previously been added did not significantly change the heights of these peaks. Cytochromes of the \(a\) type were not observed in these preparations.

\textit{Table 2. Effect of shaking rates upon oxygen uptake and acetylene reduction by Azotobacter chroococcum}

<table>
<thead>
<tr>
<th>Amplitude (cm.)</th>
<th>Sulphite oxidation value, m-mole O\textsubscript{2} l./hr</th>
<th>Oxidation absorbed, (\mu\text{moles in 40 min.})</th>
<th>Ethylene formed, (\mu\text{moles in 40 min.})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>4.25</td>
<td>32</td>
</tr>
<tr>
<td>1.7</td>
<td>9</td>
<td>12.7</td>
<td>74</td>
</tr>
<tr>
<td>4.7</td>
<td>17</td>
<td>23.1</td>
<td>645</td>
</tr>
<tr>
<td>7.9</td>
<td>22</td>
<td>25.2</td>
<td>5</td>
</tr>
</tbody>
</table>

10 ml. of culture containing 0.015 mg. bacterial protein/ml. was injected into a manometer flask containing glucose in the main compartment and KOH in the centre well under argon-oxygen (4:1) containing 4\% acetylene at 30\(^\circ\). The shaking rate was 90 strokes/min.; gas-uptake measurements were started after 2 min.

\textit{Effect of oxygen and nucleotides on acetylene reduction}

\textit{Whole bacteria.} Oxygen uptake and acetylene reduction were measured at four shaking rates giving sulphite oxidation rates (Cooper, Fernstrom & Miller, 1944) between 5 and 22 mmoles O\textsubscript{2} l./hr. (Table 2). While oxygen uptake increased at each shaking rate, acetylene reduction first rose and then dropped to zero at the highest shaking rate. In a second experiment, bacteria tested at the highest shaking rate produced no ethylene, but on changing to the second highest shaking rate acetylene reduction started immediately (Fig. 3). ATP or ADP had no effect upon acetylene reduction by whole organisms.

\textit{Organisms treated with lysozyme and EDTA.} These preparations behaved as did whole bacteria when subjected to different shaking rates in the absence of added nucleotides. In addition, oxygen uptake and acetylene reduction were inhibited by ATP (Fig. 4): at the higher shaking rate (sulphite oxidation rate of 17m-moles O\textsubscript{2} l./hr) ATP partly inhibited oxygen uptake but completely inhibited acetylene reduction (Fig. 4a); at the lower shaking rate (sulphite oxidation rate of 9 m-moles O\textsubscript{2} l./hr)
neither of these parameters was much affected (Fig. 4b). ADP, on the other hand, occasionally stimulated oxygen uptake, but in most experiments it had no effect even when the same concentration of ATP completely inhibited oxygen uptake. ADP had no effect on acetylene reduction. Equimolar ADP, when added with ATP, partially prevented the inhibition by ATP of oxygen uptake and occasionally partly restored acetylene reduction. The ADP used in these experiments usually contained 10 to 20% AMP (judged by visual survey of thin layer chromatograms under ultraviolet light); however, increasing the amount of ADP did not significantly alter the situation: AMP was ineffective in comparable experiments.

**Fig. 2**

Effect of ATP or ADP on oxygen uptake with disrupted preparations from *Azotobacter chroococcum*. Conditions: NADH (10 μmoles) tris buffer (pH 7.4) (80 μmoles) and nucleotides (10 μmoles) were incubated in water in the electrode cup at 30° for 5 min. Enzyme protein (0.3 mg.) in tris buffer, bovine serum albumin, sucrose, MgCl₂, mixture (0.5 ml.) was incubated at 30° in a water bath for 5 min. and added to start the reaction. ——— Control or control + ATP + ADP; ——— control + ATP; ——— control + ADP.

**Fig. 3**

Effect of changing the shaking amplitude on oxygen uptake and acetylene reduction in *Azotobacter chroococcum*. Conditions: as described under Table 2 using the two highest amplitudes (4.7 and 7.9 cm.). After 40 min. the amplitude of 7.9 cm. was reduced to 4.7 cm.

O₂ uptake at amplitude 7.9 cm.; O₂ uptake at amplitude 4.7 cm.; ethylene produced at amplitude 7.9 cm.; ethylene produced at amplitude 4.7 cm.

**Disrupted preparations.** Acetylene reduction by these preparations was low and variable and the effect of ATP was inconclusive.

**Effect of cell treatment and carbon source.** When bacteria were treated with hyaluronidase (50 μg./ml.) sodium lauryl sulphate (0.1% w/v) or sodium deoxycholate (0.1% w/v) under conditions similar to those used with lysozyme and EDTA, acetylene reduction was enhanced 3- to 4-fold over an untreated control. However, only bacteria that were treated with hyaluronidase or sodium lauryl sulphate showed inhibition of acetylene reduction by ATP. The concentration of carbon substrate in the growth medium influenced the concentration of EDTA necessary to allow ATP inhibition of
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acetylene reduction: organisms grown with 2.5 g. and 5 g. mannitol/l. needed 0.005% and 0.01% respectively; organisms grown in batch culture with 20 g. sucrose/l. needed 0.02% EDTA.

Fig. 4. Effect of ATP on oxygen uptake and acetylene reduction by Azotobacter chroococcum treated with lysozyme and EDTA when shaken at two different amplitudes. Conditions: as described under Fig. 1a and using shaking amplitudes of 4.7 cm. (Fig. 4a) and 1.7 cm. (Fig. 4b).

DISCUSSION

Adenine nucleotides and respiration in Azotobacter. In relatively intact preparations of A. chroococcum, i.e. organisms treated with lysozyme and EDTA, ATP showed some of the properties to be expected if it is involved in a control process analogous to respiratory control in mitochondria; it inhibited oxygen uptake and its action was antagonized by ADP. The converse effect, stimulation of oxygen uptake by ADP was not unequivocally demonstrated. The absence of an effect of added phosphate ions upon oxygen uptake could be because phosphate was already present in the test medium. These experiments provide some evidence that ATP and ADP exert a control analogous to that in mitochondria; convincing evidence for the analogy would require experiments with more highly disrupted bacterial preparations: attempts to obtain such preparations were unsuccessful and are not reported here. If the principle of ATP/ADP control in A. chroococcum is accepted the question which then arises is how does ATP inhibit oxygen uptake in relatively intact A. chroococcum? In preparations obtained by disrupting bacteria suspended in bovine serum albumin, sucrose and MgCl₂ in a French pressure cell, ATP affected oxygen uptake in three different ways: it inhibited (a) glucose-6-phosphate dehydrogenase; (b) isocitric dehydrogenase and (c) succinoxidase and, to a lesser extent, the NADH oxidase system. Any of these systems would be affected by ATP in more intact preparations and the relevant one for respiratory control would depend on whichever system was rate-limiting to oxygen. A fourth possibility arises from the work in part 2. ATP markedly inhibited nitrogenase activity in bacteria treated with lysozyme and EDTA, so one could argue that this was

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the primary effect of ATP; that inhibited nitrogenase might, in some way, lead to reduced oxygen uptake. This possibility is not unreasonable if respiration is regarded as partly concerned with protecting nitrogenase from oxygen damage (Dalton & Postgate, 1969) but is made unlikely because (i) at a high shaking rate nitrogenase was completely inhibited but oxygen uptake increased over that at a lower shaking rate (see Table 2); (ii) ATP did not inhibit acetylene reduction or oxygen uptake in conditions where the oxygen solution rate was low, suggesting that the effect of ATP was not primarily upon acetylene reduction. Control via glucose-6-phosphate dehydrogenase is attractive because this is a key enzyme in Azotobacter; sugars are metabolized through the hexose monophosphate shunt and the Entner–Douderoff pathway (Mortenson & Wilson, 1954; Johnson & Johnson, 1961; Still & Wang 1964). However, the inhibition by ATP of oxygen uptake in relatively intact preparations is unlikely to be due to inhibition of glucose-6-phosphate dehydrogenase since there was no comparable inhibition by ADP. NADH oxidase may be the relevant site because so many dehydrogenases are linked to this enzyme and it was one of the most active dehydrogenases in A. chroococcum. In disrupted preparations it was apparently inhibited by ATP only during the first minute of oxygen uptake; this could be because ATPase rapidly produced sufficient ADP to relieve ATP inhibition; the prolonged inhibition by ATP in the presence of KF supports this suggestion. However, ATPase activity was not observed in preparations treated with lysozyme and EDTA, so if inhibition does occur through NADH oxidase one must make the additional assumption that the sites of ATPase and ATP inhibition are physically separated in these preparations. ATP inhibition of oxygen uptake was more often observed than stimulation by ADP and significant reversal of inhibition occurred only with equimolar amounts of ADP. These observations are in sharp contrast to respiratory control experienced in mitochondria and make it unlikely that the two systems are similar.

Control of aerobic nitrogen fixation. Accepting that acetylene reduction is a measure of nitrogen-fixing ability of the population studied, then the inhibition of acetylene reduction at the highest oxygen solution rate agrees with the evidence of Dalton & Postgate (1967) that high oxygen tensions inhibit nitrogenase activity. If the primary effect of ATP in organisms treated with lysozyme and EDTA is upon oxygen uptake rather than on nitrogenase as was argued earlier, then ATP inhibition of acetylene reduction was a consequence of its lowering the rate of oxygen uptake; this view agrees with the proposal by Dalton & Postgate (1969) that oxygen uptake protects against oxygen inhibition of nitrogenase. Nitrogenase activity in cell-free extracts of Azotobacter chroococcum can be damaged by exposure to oxygen (Kelly, 1969) and this damage is not reversed by replacing the oxygen with argon; however, oxygen inhibition of nitrogenase in whole bacteria or in organisms treated with lysozyme and EDTA was reversed without lag by reducing the oxygen solution rate. This indicates that the nitrogenase was undamaged, though inactive, at high oxygen tensions, and that synthesis of new nitrogenase was unnecessary for restoration of acetylene reduction at lower oxygen tensions. These findings support the suggestion made by Dalton & Postgate (1969) that a second, perhaps physical, nitrogenase-protecting mechanism exists which causes the enzyme to become ‘switched off’ at high oxygen tensions.

I thank Professor J. R. Postgate for useful discussion and for reviewing the manuscript and Mrs Jenny Easton for technical assistance.
REFERENCES


